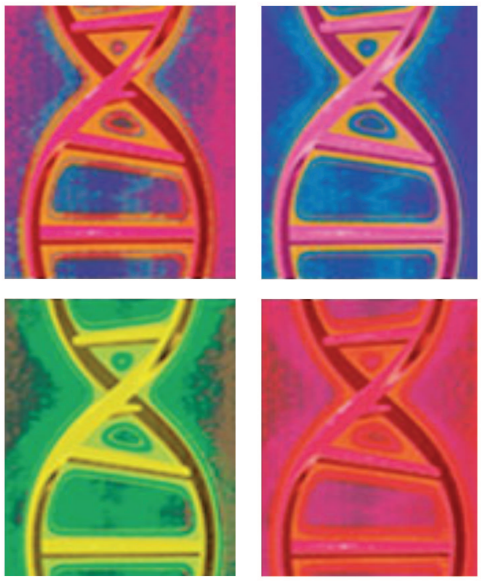
UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA
BIOCHIMICA E BIOLOGIA MOLECOLARE E CELLULARE
XXII CICLO

Genomic imprinting defects in the growth disorder Beckwith-Wiedemann syndrome



Dottoranda: Valentina Citro
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Genomic imprinting defects in the growth disorder Beckwith-Wiedemann syndrome

Valentina Citro

Relatore
Prof.ssa Maria Vittoria Cubellis

Coordinatore
Prof.Giusepe D'Alessio

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Chapter 1

INTRODUCTION

I.1 Genomic imprinting: definition

The term imprinting relates to the ability of cells to maintain a different epigenetic memory on parental chromosomes. The epigenetic memory defines the heritable modifications on chromosomal regions that do not entail a change in DNA sequence.

Epigenetic marks can regard remodelling of the chromatin, modifying of the histone proteins, DNA methylation, regulation of polycomb group protein and epigenetic function of non-coding RNA. The differential marking on the chromosome influences the transcription of the genes and the replication timing of the two parental alleles.

Genes showing differences in transcriptional activity based on parent of origin are termed as imprinted genes.

The majority of imprinted genes are organized in clusters that are structurally conserved between mouse and human.

These gene clusters are regulated by imprinting control regions (ICRs or Imprinting centres, ICs) that show different epigenetic modifications on maternal and paternal alleles (Reik W et al, 2001).

These regulatory imprinting centres are enriched in CpG dinucleotides and often constitute CpG islands that are differentially methylated on the maternally and paternally derived chromosome.

I.2 Epigenetic modifications

The two major mechanisms, sometimes strictly interdependent, in the epigenetic regulation of genes involve changes in the structure (remodelling) of

chromatin, through covalent modifications of histone proteins and DNA methylation.

In an eukaryotic cell, the chromosomal DNA is condensed and compacted with histone proteins. The condensed chromatin structures are capable of folding and unfolding transitions that determine the accessibility of regulatory factors to the DNA and, subsequently, gene activity. Different epigenetic modifications on DNA or on histone tails modulate the folding and unfolding transitions.

An extensive literature shows an elaborate collection of post translational modifications such as acetylation and methylation of lysine (K) and arginine (R), phosphorylation of serine (S) and threonines (T), ubiquitylation and sumoylation of lysine, as well as ribosylation that take place on the tail domains of the histones.

In addition, each lysine residue can accept one, two or even three methyl groups, and an arginine can be mono or di-methylated.

So, distinct histone modifications, on one or more tails, can act sequentially or in combination, correlating with a particular biological function. For example, a transcriptional inactive state is characterized by histone deacetylation at Lys-14, which precedes methylation at Lys-9 (Noma, Allis et al. 2001). In contrast, the active transcriptional state has a combination of H3 K14 acetylation and H3 S10 phosphorylation (Lo, Duggan et al. 2001).

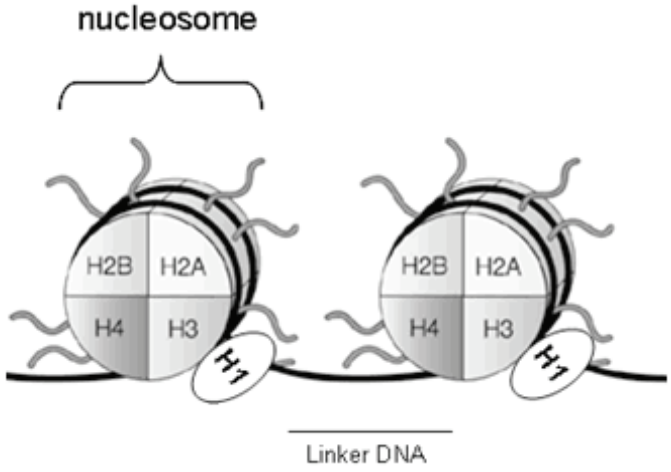


Figure 1. Structural organization of nucleosomes in chromatin

Another known epigenetic modification that can alter the chromatin, is on chromosomal DNA. Genomic DNA can be modified on nucleotides as methylated cytosines or methylated adenines (e.g. plant, bacteria).

Methylation tags cytosine, one of the four chemical bases that make up the genetic code, with a methyl group at C5-pyrimidine position. Methylation of DNA at cytosine-guanine (CpG) dinucleotides is found in virtually all vertebrates, many invertebrates, and many plant species (although these also methylate other nucleotides; ref. Lyko *et al.*, 2000). Most CpG are located in CG-rich stretches of DNA known as CpG island. Methylation of the CpG island prevents the recruitment of transcriptional factors to the promoter and as a result the associated promoter is stably silent.

In the imprinted genes, the CpG rich regions are differentially methylated on the maternally and paternally derived chromosome and so called differentially

methylated regions (DMRs). Methylation of silencer or insulator elements blocks the binding of the cognate binding protein.

I.3 Establishment and propagation of DNA methylation

Genomic imprinting in mammals determines parental-specific (monoallelic) expression of a relatively small number of genes during development. In order to preserve this phenomenon from one generation to another, imprints should be:

1) Erased during germ cell differentiation.
2) Established according to individual's sex and maintained for the rest of the life.
3) Faithfully transmitted from one cell division to another.

Erasure: Epigenetic marks are reprogrammed in order to ensure that every generation receives the appropriate sex specific imprint.
Parental epigenetic imprints are erased in primordial germ cells (PGCs) before being reprogrammed according to the type of the gamete. Demethylation in PGCs starts at around embryo day 10 (E10) and is completed by day E12 (Szabo and Mann 1995).

Establishment: After erasure, de novo methylation begins in both germ lines. The Dnmt3 family mainly constitutes the de novo methyltransferase enzymes. Among Dnmt3 family proteins, Dnmt3a and Dnmt3b are the active de novo methyltransferases and are expressed in male and female germ cells (Okano, Bell et al. 1999; Lucifero, Mann et al. 2004). Dntmt3L is the first factor known

to be involved in the establishment of primary imprints during gametogenesis. Establishment of primary methylation imprints occurs during late differentiation of the gametes. These primary imprinting marks are epigenetic modifications that are established in the gamete and that keep the parental epigenetic memory of the alleles in somatic cells.

Maintenance: Immediately after fertilization a global demethylation event is observed. In the zygote, the paternally derived genome in mice (also in other mammalian species with some differences) is active demethylation and most of the methylation marks established during spermatogenesis are eliminated. (Mayer, Niveleau et al. 2000; Oswald, Engemann et al. 2000; Santos, Hendrich et al. 2002). In contrast, maternally derived genome retains DNA methylation during this process, but subsequently also undergoes a passive demethylation during cell divisions due to the absence of the maintenance methyltransferase until the blastocyst stage (Rougier et al.1998; Reik et al. 2001).

During this demethylation event, most of the imprinting centres and some repetitive elements faithfully maintain the DNA methylation state (Olek and Walter 1997; Reik, Dean et al. 2001).

The DNA methylation marks are faithful transmitted from one cell to another during mitosis. This activity is mediated by Dnmt1 enzyme, that preferentially methylates at hemi methylated sites of double stranded DNA. It is recruited at the replication fork machinery during DNA replication and methylates the newly formed daughter strands depending on the epigenetic state of the parent template (Leonhardt, Page et al. 1992).

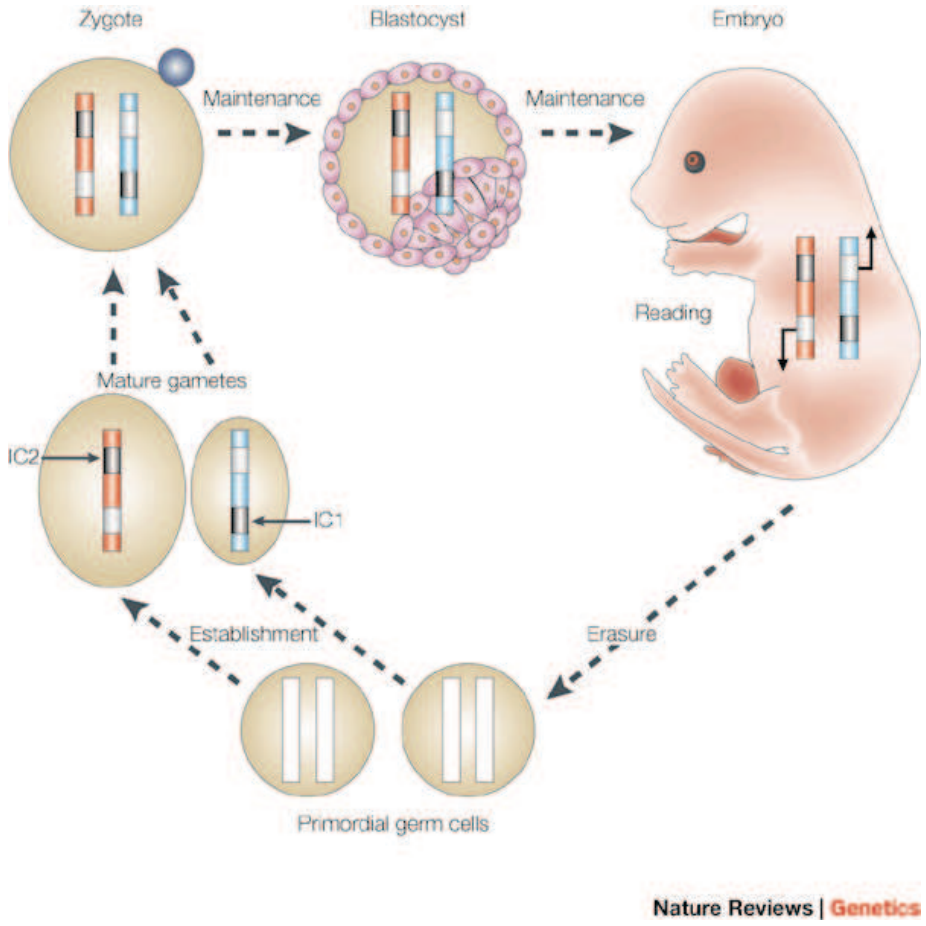


Figure 2. DNA methylation imprint establishment and propagation

I.4 Imprinting centres and their regulation

Imprinted genes are organized in clusters and are regulated by one or more Imprinting Control Regions (ICR). Different epigenetic mark on parental alleles defines the differential regulation of the imprinting centres.
Imprinting centres behave differently in their mechanisms of action; among them some are well understood while others are still under investigation.
In the majority of cases these centres function either as silencers or as insulator/boundary elements.
The disruption of the balance of epigenetic networks can cause several pathologies, including cancer and syndromes involving chromosomal instabilities and mental retardation (Arnaud P et al, 2005).
The human overgrowth and tumour associated disorder BWS provides a paradigm for investigating imprinting in human disease.

I.4.1 Imprinting centres in the Beckwith-Wiedemann syndrome (BWS) region

The human chromosome 11p15.5 and its homologous region on the mouse distal chromosome 7 harbour a cluster of imprinted genes. This cluster, which is well studied in the mouse, is functionally subdivided in two independent regions with an imprinting centre each (IC1 and IC2, Cerrato et al., 2005).

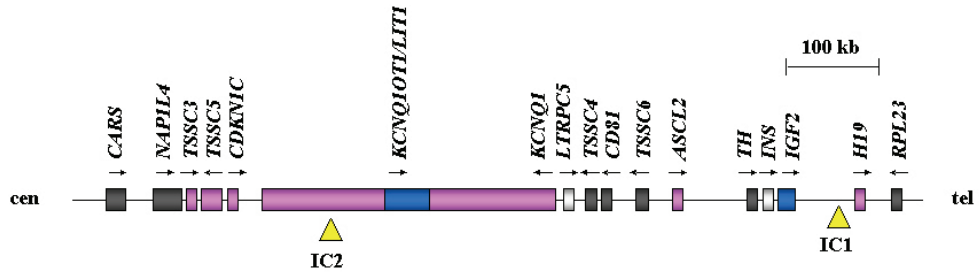


Figure 3. Imprinted gene cluster on chromosome 11p15.5

The cluster is divided into two domains, regulated by specific imprinting centres (yellow triangles). Pink rectangles indicate maternally expressed genes, blue rectangles paternally expressed genes, black rectangles biallelically expressed genes and white rectangles gene with an imprinted expression not precisely defined. Arrows above each gene indicate the direction of transcription.

I.4.1.1 Imprinting centre 1 (H19 DMR)

Domain 1 contains the two genes *IGF2* and *H19* and is located on the distal end (telomeric) of the 11p15.5 region. *IGF2* is a paternally expressed embryonic growth factor whereas *H19* is a maternally expressed gene encoding a biologically active non-translated mRNA of unknown function. The imprinted expression along the *IGF2/H19* locus is regulated by an IC located upstream of *H19* (IC1 or *H19* DMR; Fig. 4).

Numerous mouse studies allowed unravelling the role of IC1, role that seems to be conserved in humans.

In the mouse, IC1 is a methylation-sensitive chromatin insulator located between Igf2 and H19 (Hark et al, 2000). Igf2 is a paternally expressed fetal

growth factor gene with an important role in cancer development; H19 is a maternally expressed non-coding RNA with possible tumour-suppressor functions.

IC1 is unmethylated on maternal allele and is methylated on paternal allele. (Tremblay, Duran et al. 1997; Ishihara and Sasaki 2002).

When H19 DMR is un-methylated, it acts as a boundary/insulator element and this function is mediated by the CTCF (zinc finger protein) protein. This binding is required on the maternal chromosome for maintaining the methylation-free status of the region and preventing the activation of the Igf2 promoter by downstream enhancers that activate the H19 gene instead.

In fact, the CTCF protein complex at H19 DMR interacts with DMR1 (located in intron of Igf2 gene) and this interaction places Igf2 gene in a silent chromatin state and inaccessible to enhancers. The enhancers downstream of H19 are now in the vicinity of the H19 gene and eventually enhance its gene transcription. On contrary to this, on the paternal allele, the H19 DMR is methylated and CTCF protein is excluded from binding to the H19 DMR. Unknown protein complex machinery binds at the methylated H19 DMR and also interacts with the methylated DMR2 at Igf2 gene.

The interaction now places H19 gene in a silent chromatin state and the enhancers in the vicinity of Igf2 promoter. This results in Igf2 gene transcription and hence Igf2 is paternally expressed. This is a good example illustrating that epigenetic mark (DNA methylation) confers allele specific effects on transcription via long-range interactions between the DMR sites (Murrell, Heeson et al. 2004).

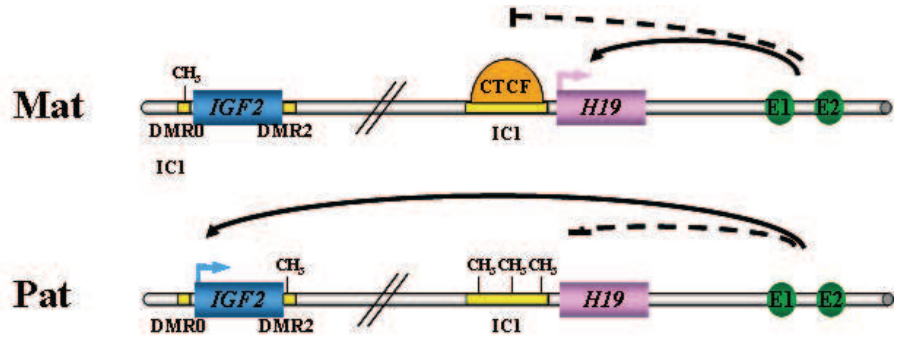


Figure 4. Domain 1

Imprinting at the human *IGF2/H19* domain is regulated by the chromatin insulator element. Three DMRs (yellow boxes) have been identified at this locus. The intergenic DMR upstream *H19* is an imprinting control element (IC) and is required for germline imprinting of both *IGF2* and *H19*. It binds the CTCF protein on the unmethylated maternal allele and this insulates the downstream enhancers (green ovals) from the *IGF2* promoters, allowing them to drive *H19* expression from the maternal allele. When methylated on the paternal allele, CTCF cannot bind to IC1 and the enhancers can drive paternal *IGF2* activity. The *H19* promoter is also a DMR, becoming methylated on the paternal allele after fertilization.

I.4.1.2 Imprinting centre 2 (KvDMR1)

The imprinting centre 2 (IC2 or KvDMR1) is a region differentially methylated on the maternal and paternal alleles. IC2 is methylated on the active maternal allele of the Kcnq1 gene and includes the promoter of the paternally expressed Kcnq1ot1 transcript. This transcript is noncoding, and overlapping antisense to the maternally expressed protein coding gene Kcnq1 and is flanked on either side by maternally expressed imprinted genes, including Cdkn1c, with

important roles in fetal and placental growth (Mitsuya, Meguro et al. 1999; Engemann, Strodicke et al. 2000).

This process appears to be very similar to the more extensive inactivation of the X chromosome. Imprinted X-chromosome inactivation occurs in the mouse placenta, by a process whereby the entire paternal X chromosome is coated by the Xist noncoding RNA expressed from the paternal IC(Jaenish et al., 2003). A general role for noncoding RNA produced at ICs may be in the recruitment of proteins and enzyme complex that modify chromatin and silence gene transcription.

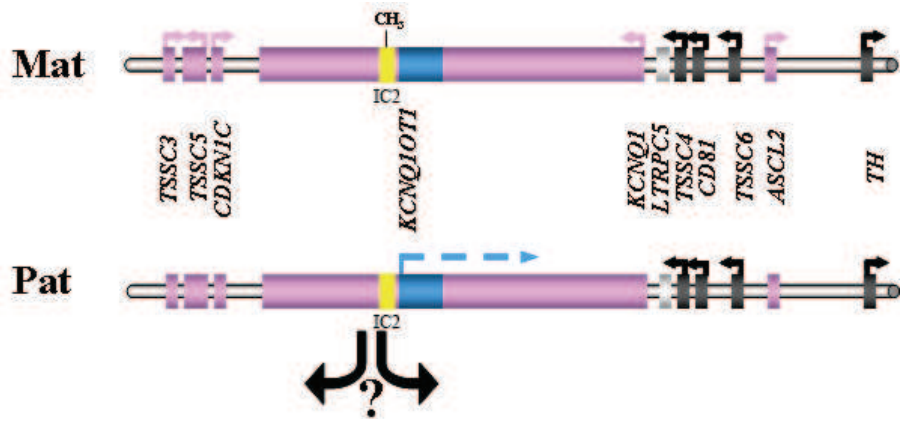


Figure 5. Domain 2

Imprinting at the *CDKN1C/KCNQ1OT1* domain is controlled by an imprinting centre (IC2; yellow rectangle) methylated on the maternally inherited allele and unmethylated on the paternal allele, located in the promoter region for an antisense ncRNA (*KCNQ1OT1*). When unmethylated, this antisense transcript is expressed and is necessary for silencing the paternal allele of *Kcnq1*, *Cdkn1c* and other flanking maternally expressed imprinted genes, by an unknown molecular mechanism.

I.5 Beckwith Wiedemann syndrome (BWS)

Beckwith Wiedemann syndrome (Online Mendalian inheritance in Man (OMIM:130650) is a clinically heterogeneous overgrowth syndrome associated with an increased risk for embryonal tumour development. It represents also a genetically complex disorder and provides unique opportunities to explore defects of genomic imprinting at 11p15.5 subchromosomal region. It was first observed by Beckwith (1963) and Wiedemann (1964). The cardinal and features of BWS are macrosomia (large body size), macroglossia, visceromegaly, embryonal tumors (e.g., Wilms tumour, hepatoblastoma, neuroblastoma, and rhabdomyosarcoma), omphalocele, neonatal hypoglycemia, and ear creases/pits. Additional diagnostic findings include polyhydramnios and premature enlarged placenta, and cardiomegaly.

BWS syndrome is sporadic in nature and the aetiology of this syndrome involves genetic and epigenetic factors.

I.5.1 BWS cases

The majority of the BWS cases are sporadic (80%). About 20% of these cases have uniparental paternal disomy (UPD) of 11p15.5 loci, with two paternally derived copies of chromosome 11p15.5 and no maternal contribution for that region, indicating that BWS is caused by excess of imprinted genes expressed from the paternal chromosome and/or defect of imprinted genes expressed from the maternal chromosome.

Although the region of UPD varies, UPD for chromosome band 11p15.5 is always present involving both Domain 1 and 2. The vast majority of patients with UPD exhibit somatic mosaicism. This implies that UPD arises post-zygotically as a result of a somatic recombination (reviewed by Weksberg, 2005). Strikingly, somatic mosaicism for UPD in BWS patients may explain the variable BWS phenotype ranging from isolated hemihyperthrophy up to complete form of the syndrome (Hoyme *et al.*, 1998). It is important to underline that although 11p15.5 UPD is clearly a genetic abnormality, its effects are epigenetics because lead to *IGF2* overexpression and *CDKN1C* down-regulation.

The rare familial cases show a predominantly autosomal-dominant inheritance and preferential expression following maternal transmission.

Only 5% of the cases (40% of the familial ones) have typical single-gene defects, consisting in loss of-function mutations of CDKN1C.

The most frequent molecular alterations in sporadic BWS majority are epigenetic alterations at either IC1 or IC2.

40% of all sporadic cases display loss of methylation (LOM) at IC2, that is associated with down regulation of CDKN1C. *CDKN1C* is both a tumour suppressor gene and a potential negative regulator of fetal growth. Developmental abnormalities, but paradoxically without net overgrowth, are seen in *Cdkn1c* knockout mouse embryos (Zhang *et al.*, 1997). However, it seems that the over-expression of *Cdkn1c* causes growth retardation in transgenic mice carrying an 800 kb YAC spanning the entire Domain 2 (Cerrato *et al.*, 2005).

These findings suggest that either an excess of paternally expressed and growth promoting gene *IGF2*, or a deficiency of maternally expressed and growth

suppressor gene *CDKN1C*, or both, are key events in the molecular pathogenesis of BWS.

Another 5-10% sporadic cases show gain of methylation at the maternal IC1 associated with biallelic activation of IGF2 and biallelic silencing of H19. The hypermethylation of the IC1 on the maternal allele blocks the CTCF binding and abolishes the enhancer blocking activity of the DMR causing the activation of the *IGF2* gene on the maternal allele. In many cases of BWS (as well as in isolated Wilms' tumours) biallelic *IGF2* expression is accompanied by monoallelic *H19* expression and normal methylation of IC1. This finding is referred to as an *H19*-independent LOI and its significance in BWS is not completely understood (reviewed by Weksberg, 2005).

BWS syndrome has also been shown in association to embryonic tumours. Among them Wilms tumour (kidney tumour) arising in BWS patients exhibit loss of imprinting with hypermethylation of IC1 at the 11p15.5 locus.

Moreover, patients with non-syndromic Wilms' tumours also have IC1 hypermethylation with biallelic activation of IGF2 and biallelic silencing of H19, but this is restricted only to cancer tissues.

Recent evidences show that genetic lesions, such as deletions of the imprinting centres 1 are also responsible of BWS.

I.5.2 IC1 microdeletions

Microdeletions of IC1 have been associated with BWS (Sparago et al., 2004; Prawitt et al., 2005).

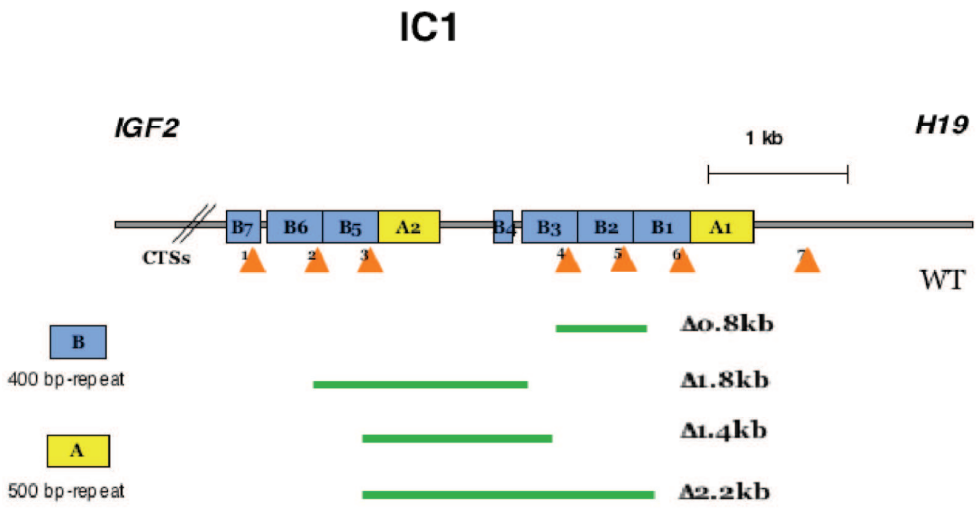


Figure 6. IC1 microdeletions

Several deletions removing part of IC1 are described

The human IC1 region (Fig. 6, GenBankTM accession number AF125183) consists of two repeat units (1 and 2) that eaach consist of an H19 proximal 459_bp direct repeat (A1 and A2) followed by several 400-bp repeats (B1-7), two of which are incomplete (B4 and B7). Repeat Unit 2 extend to 5.3 Kb upstream of the H19 transcription start and is separated from Repaet Unit 1 by 387 bp of unique sequence. The 450-bp motifs A1 and A2 are 84,5% identical. The 400-bp motifs B1,B2; and B3 of repeat Unit 1 as well as B5 and B6 of Repeat Unit 2 show a remarkable 85-91% identy to each other.

Each of the B-repeats contains a target site for the protein CTCF (CTS).

CTCF is a multi-zinc finger protein that is able to dimerize and to protect its binding site from de novo methylation.

1.4 and 1.8 Kb deletions of the IC1 region with gain of methylation at ICR1 were found. The deletions remove 1-2 target sequence for CTCF (CTS) resulting in hypermethylation of the residual CTCs and cosegregate with the BWS phenotype only if the deletion is maternally inherited (Sparago et al., 2004).

Due to its repetitive structure, the microdeletions of the human IC1 region are not rare, in fact a shorter deletion of 800 Kb was fortuitously identified in two related normal individuals (unpublished).

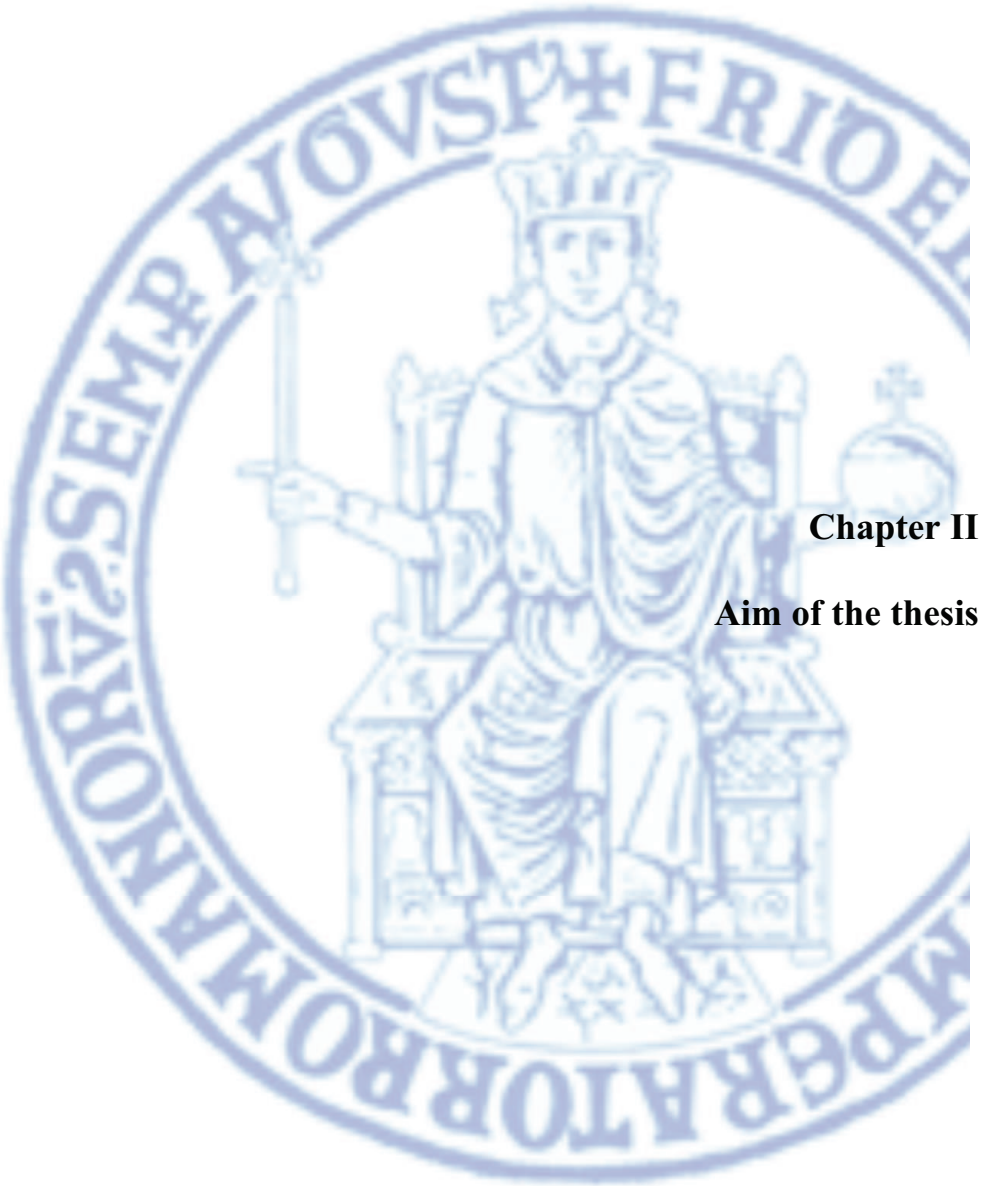
A larger deletion (2.2 Kb) removing 3 CTCs was reported by Prawitt et al. (2005). In this case, maternal transmission of this deletion was not necessarily associated with the BWS phenotype. Indeed, an additional mutation consisting in duplication of the 11p15.5 region was present in the affected children. Interestingly, this deletion did not alter the methylation of the flanking sequences, suggesting that the hypermethylation of the imprinting centre significantly contribute to the BWS pathogenesis.

It was observed that the mutant alleles with gain of methylation (1.4 and 1.8 Kb deletion) disrupt the normal element IC1 structure by creating abnormally spaced CTCs, while the normal 3 CTS-cluster organization is maintained by the 2.2 deletion.

Table 1 Different types of deletions in the human IC1 region

IC deletion	Extention (bp)	Break-point range*	Fused repeat
0.8	813	6899-6941/7712-7754	B3/B1
1.8	1834	5297-5314/7131-7148	B6/B3
1.4	1433	5723-5752/7156-7185	B5/B3
2.2	2245	5710-5721/7955-7966	B5/B1

* GenBank Ac.No. AF125183



Chapter II

Aim of the thesis

DNA methylation at the imprinting centres is the main regulatory modification of the imprinting domain.
The human overgrowth and tumour associated disorder BWS provides a paradigm for investigating imprinting in human disease.
Deletions removing part of IC1 have been found in patients affected by BWS. These mutations result in the hypermethylation of the remaining IC1 region, loss of IGF2/H19 imprinting and fully penetrant BWS phenotype when maternally transmitted.

In my present thesis I am addressing two questions:

1) Influence of mutations on the IGF2/H19 imprinting control region in the Beckwith-Wiedemann syndrome

To investigate the mechanisms by which these mutations influence the epigenetic status and function of the insulator, we have set up a cell culture system in which the different human IC1 alleles are transfected and analysed.

2) Mechanism and timing at which imprinting defects occur in patients with hypermethylation at IC1 and no accompanying deletion.

We have investigated with more detail the DNA methylation of IC1 in patients with hypermethylation at IC1 and no accompanying deletion to verify if methylation defect involves the entire IC1 region, and the timing at which imprinting defects occur by determining the grandparental origin of the chromosome carrying the methylation defect in these BWS patients

Chapter III

Results

III.1 Influence of mutations on the IGF2/H19 imprinting control region in the Beckwith-Wiedemann syndrome

The parent of origin-dependent expression of the IGF2 and H19 genes is controlled by the imprinting centre 1 (IC1) consisting in a methylation-sensitive chromatin insulator.

As discussed earlier in the introduction, deletions removing part of IC1 have been found in patients affected by BWS. (Sparago et al., 2004,2007; Prawitt et al., 2005)

Only some of these deletions (1.4 and 1.8 Kb deletions) result in the hypermethylation of the remaining IC1 region, loss of IGF2/H19 imprinting and fully penetrant BWS phenotype when maternally transmitted and some do not, suggesting that the spacing or the type of target sites deleted is relevant for the phenotypic effect.

In order to investigate the mechanisms by which these mutations alter the function of the IGF2/H19 imprinting control region in the Beckwith-Wiedemann syndrome, we have set up a cell culture system in which the different human IC1 alleles are transfected and analysed.

III.1.1 Enhancer-blocking activity

The human IC1 region consists of two types of repeats (A and B). Two clusters of three B-repeats are followed by a single repeat. Each of the B-repeats contains a target site for the protein CTCF (CTS) embedded in 400 bp sequence.

CTCF is a multi-zinc finger protein that is able to dimerize and to protect its binding site from de novo methylation.

Hence I was interested to investigate the mechanisms by which mutations on the IGF2/H19 imprinting control region influence the epigenetic status and function of the insulator.

To test whether the mutations on the IGF2/H19 imprinting control region affected insulation, we performed an insulation assay using a reporter construct in which the wt and microdeletions of the human IC1 regions are placed between the SV40 enhancer (E) and a luciferase (L) expression cassette controlled by the SV40 promoter (p). In total, we tested four different sequences derived from the normal and deleted human IC1 alleles (Fig. 12).

After linearization, these constructs were co-transfected into NIH3T3 cells with the PGK-neo vector that renders cells resistant to neomycin; stably transform mammalian cells were screened by neomycin selection and their luciferase activities were determined.

If the test sequence has no enhancer-blocking activity, luciferase expression is fully activated. In contrast, if the test sequence harbours an enhancer blocker, luciferase expression is shielded from the enhancer.

The luciferase activities in the cells transfected with EWTpL were approximately two-fold lower than those in cells transfected with EpL (control vector). A similar reduction in luc activity was seen in cells transfected with the vectors of 0.8 and 2.2 Kb deletions of the human IC1 regions (E0.8pL, E2.2pL) while the E1.4pL produced no comparable reductions in luc activity (Fig. 7). Luc activity was expressed as RLU/mg of protein.

Our observations show that the 1.4 Kb deletion lost enhancer-blocking activity.

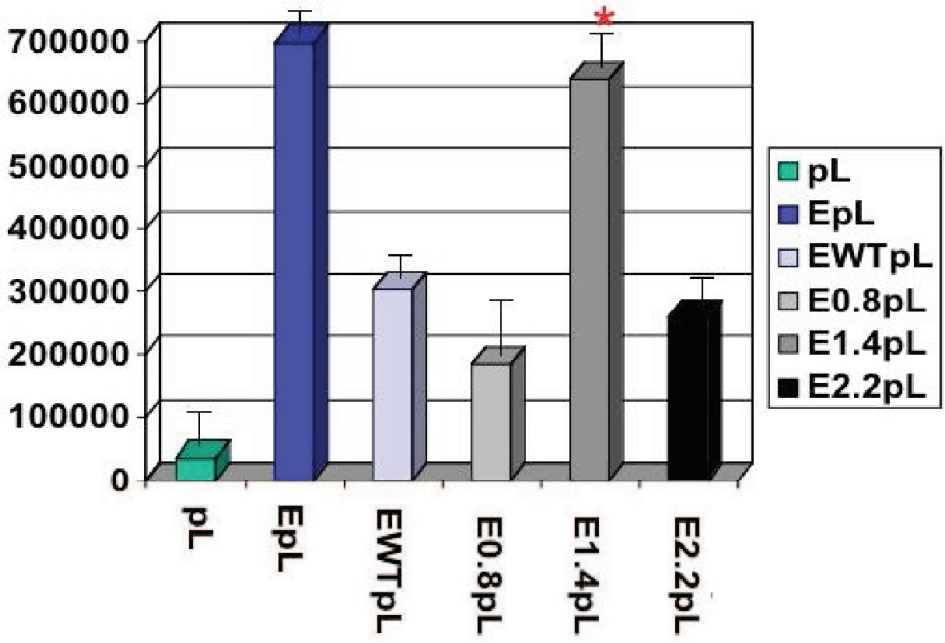


Figure 7. Enhancer-blocking activity
Reporter gene constructs used for analysis for enhancer-blocking test. NIH3T3 cells transfected with each construct were selected using neomycin. Luc activity is expressed as RLU/mg of protein. Note that cells transfected with E1.4pL exhibit remarkably high degree of luc activity.

III.1.2 CTCF binding

The next task was to check the binding of CTCF protein to the different microdeletion alleles. For this purpose, we employed the Chromatin Immuno-Precipitation (ChIP) assay in stably transformed cells.

After formaldehyde-based crosslinking of protein and DNA, chromatin fragmented by sonication was immunoprecipitated with anti-CTCF antibody,

followed by RT-PCR amplification using specific primers for the IC1 region and for the promoter of the amyloid precursor protein gene. To directly compare the CTCF binding levels obtain from the different IC1 sequences, the values of immunoprecipitation are divided by the signal derived from the positive sequence control (APP), assuming that the CTCF binding level at the promoter of the APP gene does not differ between samples, and that consequently all signal variation are due to different binding of the protein CTCF (Fig. 8 A, B).

A similar CTCF binding was seen in cells transfected with the vectors of wild type (WT) and 0.8 ; 2.2 Kb deletions of the human IC1 regions (EWTpL, E0.8pL, , E2.2pL) while the E1.4pL showed a reductions in the CTCF binding (Fig. 9).

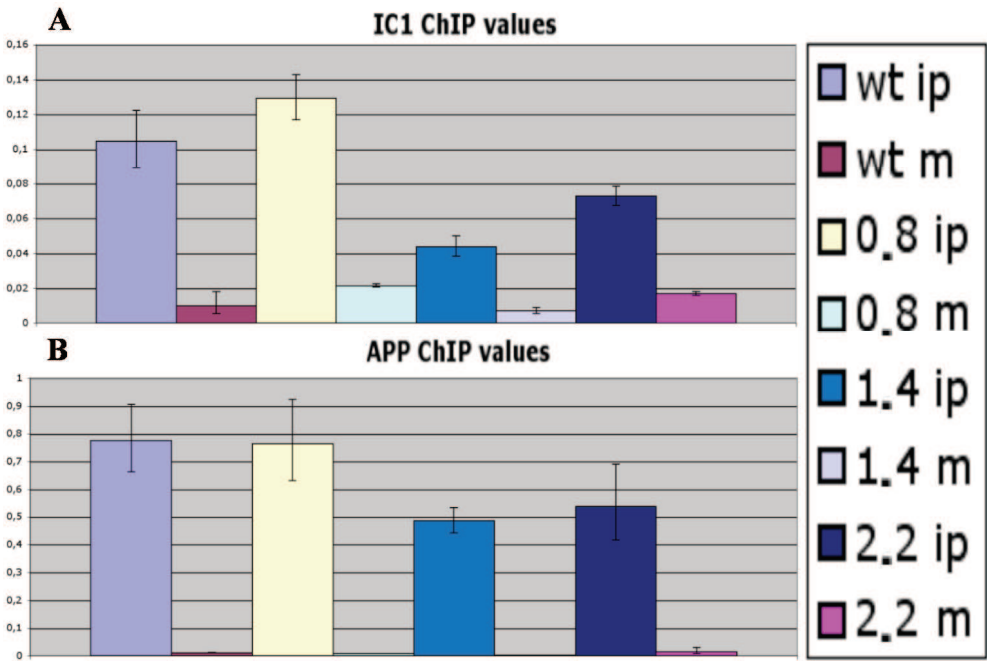


Figure 8. CTCF binding
ChIP in NIH3T3 cells transfected stably with wt and microdeletion IC1 constructs. (A) Levels of CTCF binding to the IC1 normal and alterated region. (B) Levels of CTCF binding to the promoter of the APP gene.

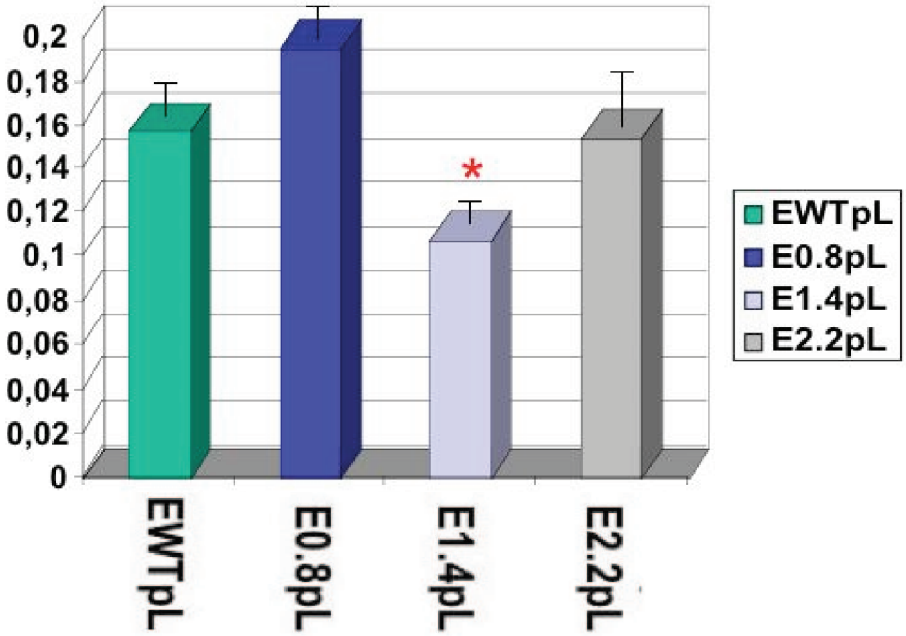


Figure 9. Immunoprecipitation efficiency
The values of immunoprecipitation efficiency derived by the levels of CTCF binding to the IC1 normal and alterated region divided the positive sequence control (APP). Note that cells transfected with E1.4pL exhibit a lower degree of binding.

III.2 Mechanism and timing at which imprinting defects occur in patients with hypermethylation at IC1 and no accompanying deletion

The imprinting centre IC1 is a primary DMR (Differentially Methylated Region) that acquires different DNA methylation mark in the germ line and maintains the difference through out the development. Methylation of the IC1 allele inhibits the binding of the CTCF to this regulatory region and allows the activation of Igf2 while H19 is silenced on the paternal chromosome. The

individual with biallelic methylation of IC1 are believed to have loss of imprinting, with biallelic activation of Igf2 and biallelic silencing of H19.

We have investigated with MR-PCR the DNA methylation of each of the 7 CTCF sites of IC1 in 12 BWS patients with hypermethylation at IC1 and no accompanying deletion to verify if methylation defect involves the entire IC1 region.

In short, MR-PCR consist in treatment of genomic DNA with sodium bisulfite, after the PCR products were subjected to the restriction enzymes digestion and later analyzed by polyacrylamide gel electrophoresis. After bisulfite treatment and PCR amplification, most of the restriction sites are "mutated" due to bisulfite induced base changes, and also a few new sites are created. Presence of methylation on cytosine however prevents the disruption of some restriction sites. The digestion pattern of PCR amplicons, obtained from methylated and unmethylated template, can be easily distinguished.

III.2.1 DNA methylation at CpG islands in the IC1

MR-PCR analysis revealed differences in the extent of methylation of the individual CTCs and H19 promoter in these patients.
Methylation was analysed in the peripheral blood leukocytes and ranged from 50 to 99% at different sites and in different patients. In addition, the degree of methylation was more homogeneous among the CTSs belonging to the same cluster and the CTSs 4–7 and H19 promoter were generally more methylated than the CTSs 1–3. Three individuals (BWS-12, BWS-19 and BWS-21) showed

this characteristic in a more pronounced manner, having 50–60% methylation at the first three CTSs and 76–90% methylation at the further four CTSs and H19 promoter.

These results indicate that, in the BWS patients without IC1 deletion, the abnormal methylation at IC1 is mosaic in the majority of the cases and can affected the entire or only the 3' half of the IC1 region.

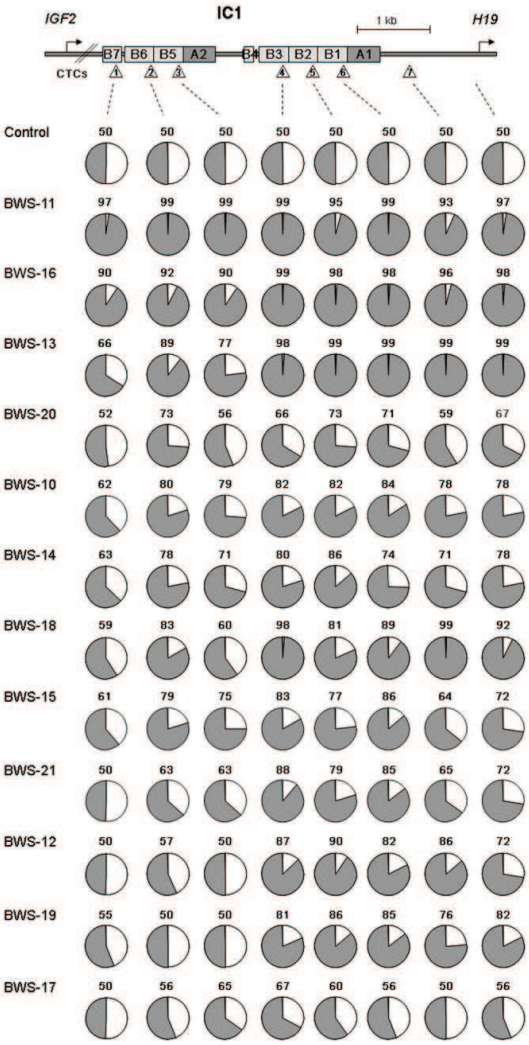


Figure 10. Methylation of the CTSs and H19 promoter in the BWS patients with IC1 hypermethylation and no associated deletion.

Summary of the results obtained on 12 BWS patients and 40 control individuals. DNA methylation at CpGs included in the CTSs and H19 promoter was assayed by MR-PCR (COBRA) in leukocyte DNA. The average methylation levels detected in control individuals (50+5%) are shown for comparison. The extent of methylation at each CpG site is indicated with pie charts filled in black. Each sample was run in duplicate. SD was ~5%. A diagram showing the structure of the human IC1 region is present in the upper part of the figure. A- and B-type repeats are indicated by boxes of different colours, whereas the CTSs are indicated by triangles.

III.2.2 Segregation of 11p15.5 haplotypes

Since in BWS the chromosome carrying the imprinting defects derives from the mother, we have analysed the segregation of the maternal IC1 allele of the index patient in the families without IC1 microdeletion after construction of 11p15.5 haplotypes by microsatellite analysis.

The results showed that in one family (BWS-20), the affected and an unaffected sib inherited the same 11p15.5 haplotype from their mother. In three additional families (BWS-11, BWS-17 and BWS-21), different 11p15.5 haplotypes were transmitted to the patients and their healthy siblings from their mothers. However, in family BWS-11, the propositus and her healthy mother shared the same 11p15.5 haplotype on the maternal chromosome. So, in at least two families, affected and non-affected individuals had the same maternal IC1 allele.

The timing at which imprinting defects occur was studied by determining the grandparental origin of the chromosome carrying the methylation defect in these BWS patients. If the grandpaternal DNA was available, this assay was performed by microsatellite analysis or (if grandparents were not available) DNA methylation/SNP test was used. In this procedure, the grandpaternal methylated and the grandmaternal non-methylated IC1 alleles were distinguished in the DNA of the mothers by bisulfite sequencing. In case of heterozygosity, DNA sequence polymorphisms (SNPs) were used to determine which maternal allele was transmitted to the probands.

The results showed that in five informative cases the chromosome carrying the imprinting alteration derived from the maternal grandfather, whereas in one case derived from the maternal grandmother.

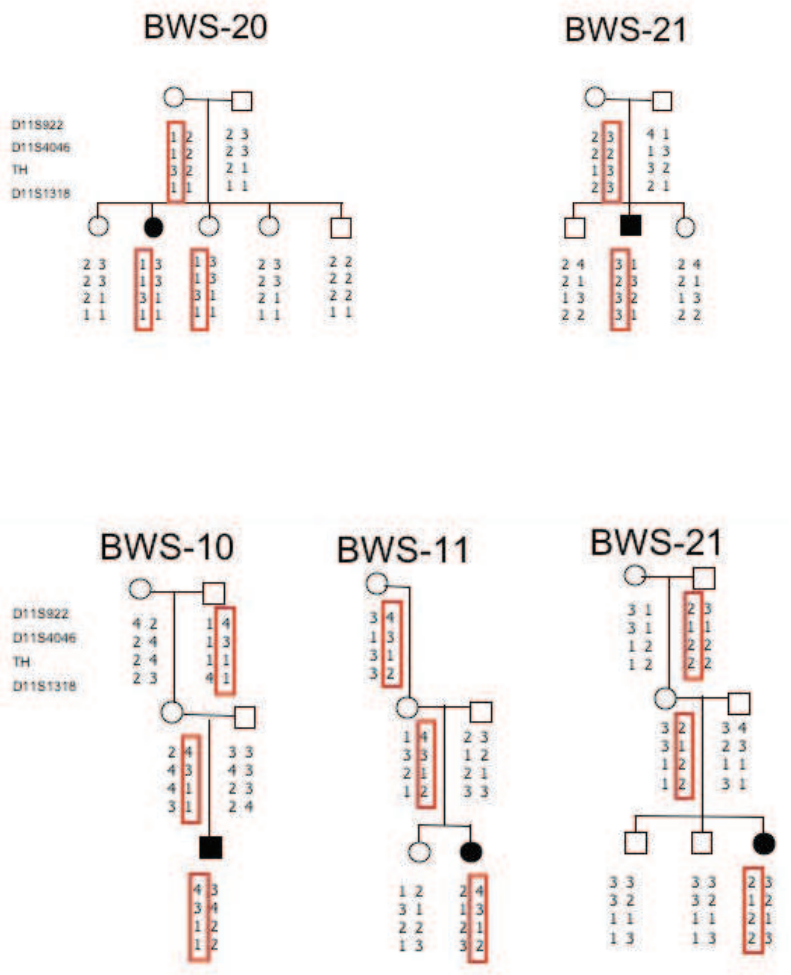
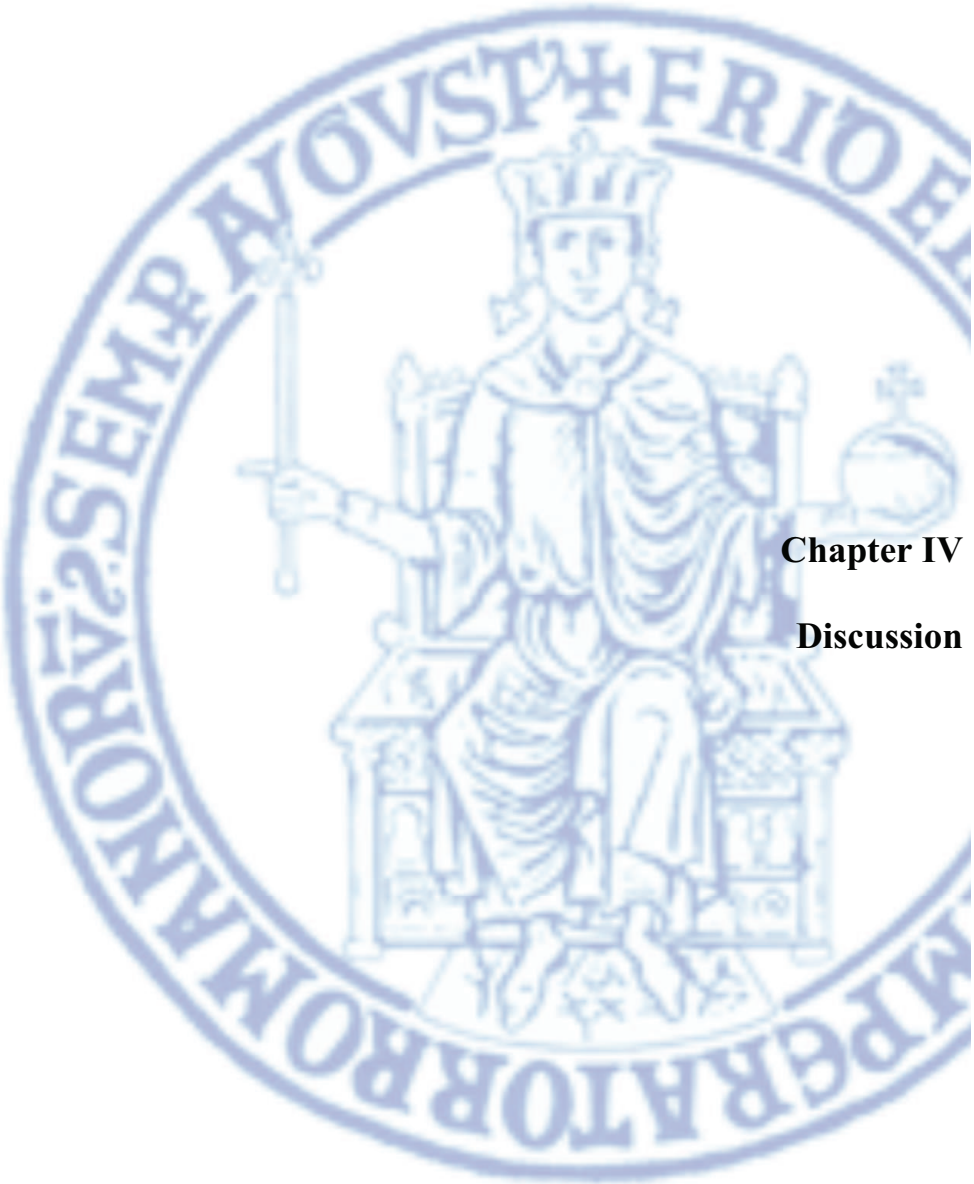


Figure 11. Segregation of chromosome 11p15.5 genotypes in BWS families with IC1 imprinting defect and no associated deletion.
Construction of haplotypes by microsatellite analysis. The haplotype associated with the imprinting defect in the patient is framed. The chromosome location of the IC1 region is between TH and D11S1318.



Chapter IV

Discussion

The imprinting centre 1 (IC1) is a methylation-sensitive chromatin insulator and presents a repetitive structure with 7 CTCF targets sites (CTSs)

Deletions removing one to two CTSs of the human IC1 result in gain of methylation while a larger deletion abolishing three CTSs does not affect the methylation of the locus (Prawitt et al., 2005 , Sparago et al., 2004 2007).

In order to investigate the mechanisms by which these mutations alter the function of the IGF2/H19 imprinting control region in the Beckwith-Wiedemann syndrome, we constructed different EpL based vectors carrying the microdeletions of the human IC1 regions between the SV40 enhancer (E) and the luciferase (pL) expression cassette. In total, we tested four different sequences derived from the human IC1. The first sequence simply reproduces the IC1 insulator containing the six CTCF binding sites (EWTpL). The second sequence differs in that it contains a smaller deletion (0.8 Kb) abolishing two CTCF binding sites (CTSs) identified in several normal phenotype (E0.8pL). The third sequence bears a 1.4 deletion abolishing one CTS (E1.4pL). Previous studies have shown that maternal transmission of this deletion cosegrates with the hypermethylation of the residual CTSs and BWS phenotype with complete penetrance while normal phenotype is observed upon paternal transmission (Sparago et al., 2007). Finally, the fourth sequence contains a larger deletion (2,2Kb) removing three CTSs (E2.2pL). In this case, maternal transmission was associated with loss of IGF2 imprinting but did not alter the methylation of the sequences flanking the deletion and was present in both affected and unaffected individuals (Prawitt et al., 2005).

After transfection of each of these plasmids with the PGK-neo vector, the cells were selected in the presence of neomycin. The resulting stably transformed cells were used to assay luc activity. Cells transfected with the EpL vectors

carrying the wt human IC1 region and the 0.8; and 2.2 Kb microdeletions (EWTpL; E0.8Pl; E2.2pL) resulted in decrease expression of the luc activities (Fig. 7).

Interestingly, E1.4pL, in which 1.4kb deletion generate abnormally longer cluster of target sites for the protein CTCF, exhibited a level of luc expression comparable in cell transfected with EpL (control vector) (Fig. 7) indicating that the 1.4 Kb deletion lost insulator activity.

CTCF binding to the maternal IC1 is essential for imprinting maintenance in somatic cells, as well as protection against aberrant de novo methylation. Our data suggest the possibility that the abnormally longer cluster of sites, due to IC1 microdeletion, reduce the affinity for CTCF and this results in gain of methylation of the locus.

We analyzed the CTCF binding to the IC1 microdeletion by a ChIP assay using an anti CTCF-antibody in stably transform NIH3T3 cells. The lower binding of the protein CTCF to this 1.4 kb deletion was confirmed (Fig. 9).

Consistent with this hypothesis, the maternal deletion of three CTCF sites (out of four) increase the methylation of remaining fourth site in the mouse H19DMR, while the deletion of only the third and fourth sites has no effect on the methylation of the locus (Drewell et al., 2000).

It is also plausible the CTCF molecules bound to adjacent sites interact and the cluster of three sites (present in the wt and 2.2 Kb deletion alleles with normal methylation) has high affinity for CTCF and are resistant to de novo methylation.

In fact, by analysing 12 patients with IC1 hypermethylation and no deletion, a detailed methylation analysis showed that the abnormal methylation at IC1 is mosaic in the majority of the cases and can affected the entire or only the 3’

half of the regulatory region (Fig.10). This suggests that the binding of the CTCF to the first and second cluster of the CTSs is independent while the binding to adjacent sites is probably cooperative.

This cooperative interaction between proteins bound to adjacent CTSs (Pant et al., 2004) may explain the more homogeneous methylation inside each cluster of CTSs.

It has been demonstrated in the mouse that the methylation of the H19 promoter depends on the methylation of IC1. In the BWS patients, the methylation status of the H19 promoter is always concordant with that of the 3' half of IC1, suggesting that the hypermethylation of the four more proximal CTCF sites is sufficient to cause hypermethylation of H19 promoter.

Imprinting defects at ICs can derive from failure of erasure, establishment or maintenance of the parental marks (Reik et al., 2001). One way to approach this problem is to investigate the grandparental origin of the chromosome with the imprinting defect (Buiting et al., 2003).

The segregation of the chromosome with the imprinting defect in the healthy relatives excludes inherited mutations in the entire chromosome 11p15.5 region of two individuals (Fig.11).

We observed that the imprinting defect derived from either the maternal grandfather or maternal grandmother chromosome. These results indicate that, in the absence of deletions, IC1 hypermethylation arises later on, during imprint establishment in the gametes or imprint maintenance in early embryogenesis.

Considering the sporadic nature of these cases, the possibility that IC1 hypermethylation occurs as consequence of stochastic events or environmental influence should also be envisaged (Jaenish 2003).

Chapter V

Material and Methods

V.1 Vectors for analysis of enhancer-blocking activity

The SV40 enhancer of the peDNA3.1 plasmid was PCR-amplified using the primers 5'-TATATGGGGTACCGCGTTAC-3' and 5'-GAGCTCGGGCGGAACTGG-3'. The PCR product, digested with KpnI and SacI, was subcloned between the KpnI and SacI XhoI sites of a pGL3 promoter (Promega) carrying a luc expression cassette. The resulting vector was sequenced for confirmation of fidelity of construction and named EpL (Fig. 7). The sequences derived from the normal and deleted human IC1 alleles were PCR-amplified using the primers 5'- gtagtggcgcgccatttcccaatg-3' and 5'-gcacaggcgcgccatcgaacatc-3', digested with AscI and subcloned into the MluI site of EpL (fig.13).

The presence of the insulator was confirmed and its orientation determined.

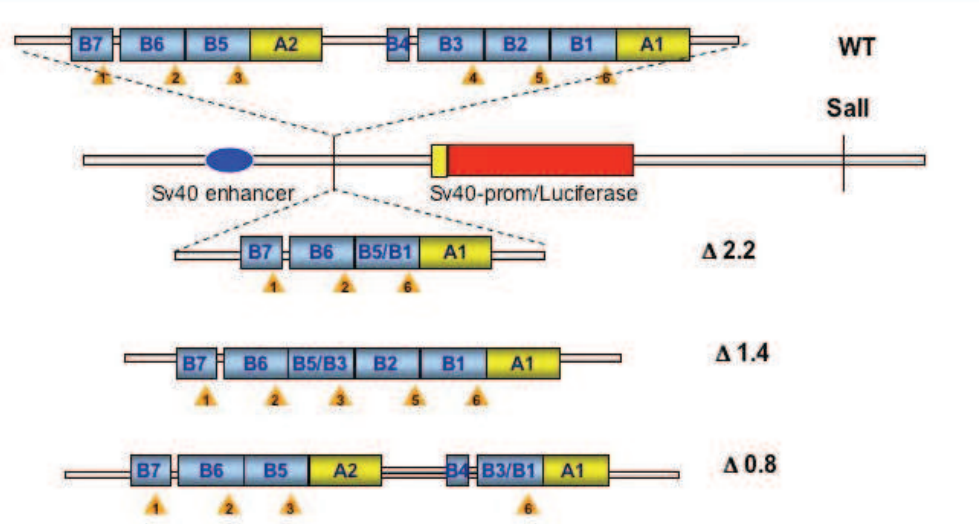


Figure 12. Vectors for the enhancer-blocking assay and ChIP assay

V.2 Cell culture and transfections

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Co. Ltd, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 50 units of penicillin and 50 microg of streptomycin per ml at 37 °C under an atmosphere of 5% CO2.

For the evaluation of enhancer-blocking activity, cells were seeded in 6 well plates (Nunc Co., Roskilde, Denmark) at a density of 1 x 105 cells per well. On the day following cell seeding, 4 microg of the SalI linearized plasmid and 0.4 microg of SalI-linearized PGK-neo vector were mixed with Lipofect Amine Plus reagent and the mixture was added to each dish. The cells were trypsinized and seeded on to 100mm plate (Nunc Co.) 48 h after cultivation. One milligram/millilitre of neomycin (Sigma Co. Ltd) was added to the medium for selection. After seven days, surviving cells were trypsinized, passed into 6-mm dishes and cultured until require for the luc assay and ChIP assay.

V.3 Luciferase assay

To test enhancer-blocking activity, luc activity was measured using the Luciferase Assay System (Promega Co.) and Dia-Iatron luminometer, following the protocols described by the manufacturers.

Firefly luc activity was normalized against protein concentration in each pool, and luc activity was expressed as RLUs per mg of protein. Protein concentrations were determined by the Bedford method (Bio-Rad, Hercules, CA, USA).

V.4 Immunoprecipitation of Chromatin

NIH3T3 cells were fixed with 1% formaldehyde for 15min at room temperature. The cells were lysed, and the chromatin was sonicated to an average size of 600 base pairs. For each experiment the 100 microgram of chromatin was immunoprecipitated with 4 microgram of the anti-CTCF antibody (Upstate), according to the manufacturer's protocols (Upstate).
The primers used to amplify the human IC1 region are TCCCGGGTCACCCAAGCCAC and AAGCCCTCGGAGTGTGACC.
The primers used to amplify the mouse promoter of the APP gene are CCCTGGAACCTTAACGTCCT and ACAGAGACCCCTAGCGGAGC

V.5 DNA methylation analyses

Methylation analyses of all seven CTCF binding sites of IC1 and *H19* promoter regions were performed by a MR-PCR that consists of bisulphite treatment and amplification, coupled with restriction enzyme digestion. After bisulphite treatment, DNA was amplified and PCR product labelled by a hot-stop cycle. Briefly, hot-stop PCR involves the addition after 35 cycles of [α-^{32}P] dGTP, allowing synthesis to take place for a final cycle (Uejima *et al.*, 2000). This method circumvents the difficulty of analysis of heteroduplex molecules because only molecules synthesized during the last amplification cycle are radio-labelled. PCR products were subsequently digested with restriction enzymes that have almost one CpG site in their recognition sequence. In Table 2 are reported primers, PCR conditions and enzymes used for the analysis of each region of the *IGF2-H19* locus.

Methylated molecules, in which CpG-cytosines were not replaced by thymines, were digested whereas in the unmethylated molecules CpG-cytosines were converted to thymines, the restriction site was lost and the enzyme was not able to cut the amplified fragment. After digestion, fragments were separated by electrophoresis on 8% polyacrylamide gels and the intensity of the bands was quantified using a PhoshorImager and ImageQuant software (Amersham).

The ratio between undigested (unmethylated) to digested (methylated) bands gave a measure of the methylation status of the site analysed

Table 2 Methylation analysis by bisulphite sequencing assay and MR-PCR: primers, PCR conditions and restriction digestion

IC1/*H19* region	PCR primer pairs	Size (bp)	$MgCl_2$ (mM)	Annealing Temperature (°C)	MR-PCR digestion
CTS1	GTATTTTTGGAGGTTTTTTATTTAG ACACCTAACCTAAAAAACCTAAAAC	231	1.5	55	*BstU*I
CTS2	AGGTGTTTTAGTTTTTTGGATGATA CCATAAATATTCTATCCCTCACTA	319	1.5	60	*BstU*I
CTS3	GGTTTTTGGTAGGTATAGAAATTG CACCTAACTTAAATAACCCAAAAC	217	1.5	62	*BstU*I
CTS4	GTTTTTGGTAGGTTTAAGAGTAAATATCCTATCCCTAATAAC	256	1.5	58	*BstU*I
CTS5	TTTTGTAGGGTTTTTGGTAGTCCCATAAATATCCTATACCTC	269	1.0	59	*BstU*I
CTS6	GAGTTTGGGGGTTTTTGTATAGTAT G CTTAAATCCCAAACCATAACACTA	337	1.5	58	*BstU*I
CTS7	GAGTATTTGTATTTTTGGAGTAT AAAAATTCTCAAACTTTTCCATAAA	253	2.5	59	*Taq*I
H19 Promoter	TGAGGGAGGTGATGGGGTAATG TTCCCCACTTCCCCAATTTCCC	390	2.0	65	*BstU*I

V.6 Microsatellite analyses

The minisatellite markers D11S922, D11S4046, Tyrosine Hydroxylase (TH) and D11S1318 were analyzed by single-strand chain polymorphism (SSCP) as follows. Purified DNA extracted from peripheral blood lymphocytes was amplified with Biotaq DNA polymerase RED (Bioline, London, UK) in a 25-µl reaction containing 200 µM dNTPs, 1 mM Mg++ (1.5 mM Mg++ for D11S1318), 20 pmoles ^{32}P-labeled forward primer, 20 pmoles reverse primer, and 2 U Taq polymerase. Primers sequences are shown in Table 3. For the D11S4046 and TH minisatellites, amplification was performed by initial denaturation (95°C, 2 min), followed by 28 cycles of denaturation (94°C for 45 seconds), annealing (60°C for 45 seconds) and extension (72°C for 45 seconds), and a final elongation step (72°C for 5 minutes). For D11S922 and D11S1318, annealing temperature was 58°C; all other parameters were the same. Two microliters of PCR reaction were mixed with 7 µl of loading buffer (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.025% bromophenol blue, 0.025% xylene cyanol) and loaded onto a 6% polyacrylamide – 8 M urea gel (6% acrylamide / bis-acrylamide 29:1, 8 M urea, 1x TBE, 0,07% ammonium persulfate, 0,05% TEMED). Gels were run by electrophoresis on 8% polyacrylamide gels and the intensity of the bands was quantified using a PhoshorImager and ImageQuant software (Amersham). The methylation values were assessed by comparing the intensity ratio of the two bands corresponding to each allele

Table 3: Sequences of primers used for microsatellite analysis

Microsatellite	PCR primer pairs
D11S1318	F: CCCGTATGGCAACAGG R: TGTGCATGTNCATGAGTG
TH	F: GTGATTCCCATTGGCCTGTTCCTC R: GTGGGCTGAAAAGCTCCCGATTAT
D11S4046	F: AGCCTGGGAAACAGAGTGAG R: GTCTCAAGCAAGCAATGTCC
D11S922	F: GGGGCATCTTTGGCTA R: TCCGGTTTGGTTCAGG

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ABBREVIATIONS

APP	Amyloid precursor protein
BWS	Beckwith-Wiedemann syndrome
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CpG	Citosyne-guanine dinucleotide
CTCF	CCCTC-binding factor
CTS	CTCF target site
DMR	Differentially methylated region
DNMT	DNA methyl transferase
MR-PCR	Methylation Restriction-PCR
IC	Imprinting centre
IGF2	Insulin-like growth factor type 2
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1
KCNQ1OT1	KCNQ1 overlapping transcript 1
LOI	Loss of imprinting
PGCs	Primordial germ cells
SNP	Single nucleotide polymorphism
UPD	Uniparental disomy

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SOMMARIO

La Sindrome di Beckwith-Wiedemann (BWS) è una patologia caratterizzata da eccesso di crescita ed aumentato rischio di sviluppare neoplasie, associata ad alterazioni in un cluster di geni imprinted presente nella regione cromosomica 11p15.5. Su tale cromosoma umano, in una regione di 1 Mb di DNA, ci sono almeno 11 geni imprinted che costituiscono un cluster genico strettamente conservato nel genoma murino, nella regione distale del cromosoma 7. I numerosi studi condotti sul modello murino hanno consentito l'identificazione di due domini funzionalmente indipendenti, ciascuno dei quali controllato da un centro dell' imprinting principale (IC1 e IC2). IC1 e IC2 corrispondono a regioni ricche in CpG che sono normalmente differenzialmente metilate (DMR) sugli alleli di origine materna e paterna ma che mostrano anomalie di metilazione nella maggioranza degli individui affetti da BWS. Il centro di Imprinting 1 (IC1) controlla l'imprinting reciproco dei geni Igf2 e H19. Igf2, espresso sull'allele paterno, è un fattore di crescita coinvolto nello sviluppo fetale mentre H19, espresso sull'allele materno, è un RNA non tradotto la cui funzione non è stata ancora chiarita. L'IC1 funziona come una barriera cromatinica (insulator), sensibile alla metilazione, localizzato tra Igf2 e H19. L'allele materno non metilato interagisce con la proteina CTCF; tale legame è necessario per impedire la metilazione della regione e prevenire l'attivazione del promotore di Igf2 da parte degli enhancers, che invece attivano il gene H19. Sul cromosoma paterno, diversamente, la metilazione del DNA previene il legame di CTCF a IC1 e permette l'attivazione di Igf2 mediata dagli enhancers mentre il promotore di H19 è ipermetilato e silenziato.
Il progetto di ricerca in cui sono stata impegnata nel periodo di tesi, si propone di studiare i meccanismi molecolari alla base dei difetti di imprinting nella BWS. A tale scopo, campioni di DNA e linee cellulari derivati da una grande casistica di individui affetti da BWS sono stati raccolti e sottoposti ad una serie di analisi, tra cui un test di metilazione dei Centri di Imprinting IC1 e IC2.
In circa il 10% dei casi è stata riscontrata l'ipermetilazione della regione IC1 che controlla l'espressione imprinted dei geni IGF2 e H19.
In alcuni di questi pazienti, tale difetto epigenetico è associato a microdelezioni all'interno del centro di controllo IC1. Queste mutazioni causano l'ipermetilazione della regione di IC1 rimanente, perdita dell'imprinting dei geni H19/IGF2, e piena penetranza del fenotipo BWS quando trasmesse per via materna. Esse si sono originate probabilmente per crossing-over ineguale tra sequenze ripetute in tandem ed hanno causato la perdita di uno o due dei sette siti di legame per la proteina CTCF, presenti nella sequenza IC1 umana.
Il meccanismo mediante il quale tali delezioni causano ipermetilazione del locus è stato oggetto di studio nel mio progetto di tesi. L'incompleta

ipermetilazione dei siti di legame per la proteina CTCF trovata nei pazienti con delezioni in IC1 da 1,4 e 1,8 Kb indica mosaicismo per il difetto d'imprinting e suggerisce che la metilazione è acquisita nella fase postzigotica e deriva da un'insufficiente protezione da metilazione de novo del mutato IC1 materno. Inoltre solo alcune delezioni sono associate a ipermetilazione, mentre altre delezioni sono state trovate in individui normali. Questi dati suggeriscono che non è il numero ma l'organizzazione dei siti di legame per la proteina CTCF ad essere responsabile del controllo delle modificazioni epigenetiche e quindi del fenotipo patologico.

Nel mio lavoro di tesi, ho studiato come le diverse strutture che la regione IC1 assume in seguito a tali delezioni influenzano il legame della proteina CTCF e la funzione di "insulator". A tale scopo, sono stati misurati il legame della proteina CTCF, mediante Chromatin Immuno-Precipitation (ChIP), e l' attività "insulator" dei diversi alleli mutati e wt, mediante l'enhancer blocking test in cellule coltivate. Le diverse sequenze derivate dai pazienti sono state clonate in vettori plasmidici, tra un gene "reporter" (luciferasi) e l'enhancer. Nell'enhancer blocking test, la trascrizione del gene è stata valutata mediante l'epressione della luciferasi in cellule NIH3T3 trasfettate stabilmente. Nelle stesse cellule, il legame della proteina CTCF è stato analizzato mediante ChIP, ovvero il complesso della proteina CTCF legato al DNA è stato immonoprecipitato e dosato mediante amplificazione con real time pcr. Nel complesso, i risultati ottenuti indicano che la spaziatura dei diversi siti di legame di CTCF nel Centro di Imprinting è determinante per l'affinità di legame della proteina e per la conseguente funzione di "insulator".

Un altro argomento di cui mi sono occupata, nel progetto di tesi, riguarda alcuni casi di BWS in cui l'ipermetilazione della regione IC1 è stata trovata in assenza di delezioni o altre mutazioni nelle sue vicinanze. In questi pazienti, la metilazione di ognuno dei 7 siti di legame per la proteina CTCF nella regione IC1 è stata analizzata per MR-PCR (Methylation Restriction-PCR). I risultati hanno dimostrato che l'ipermetilazione della regione IC1 non è uniforme. In alcuni individui, la regione IC1 è metilata in tutta la sua estensione, in altri è normalmente metilata nella sua prima metà, ma ipermetilata nella seconda parte. Inoltre, nella maggior parte dei casi, l'ipermetilazione non è presente su tutte le molecole di DNA, ma solo su alcune di queste. Un'accurata analisi familiare, ha permesso inoltre di dimostrare che in questi casi il difetto di metilazione non è associato al genotipo della regione 11p15.5 e che il cromosoma con il difetto di metilazione deriva o dalla nonna, o dal nonno materno. Il difetto di imprinting pertanto insorge de novo, come evento stocastico o conseguenza di un difetto genetico che agisce in trans.

Nel complesso, questi dati suggeriscono l'esistenza di differenti meccanismi nell'acquisizione del difetto di imprinting genomico. Alcune microdelezioni

alterano la spaziatura dei siti bersaglio per CTCF in maniera tale che l'affinità per tale proteina viene ridotta e la funzione del centro di imprinting alterata. Invece, nei casi in cui la sequenza è immutata, è possibile che il difetto colpisca un fattore che agisce in trans e che è essenziale per il mantenimento dello stato non metilato. In entrambi i casi, la copia materna di IC1 subisce, verosimilmente nelle prime fasi dello sviluppo embrionale, una metilazione de novo, che inibendo stabilmente il legame di CTCF, causa il difetto di imprinting.

Alcuni dei risultati ottenuti sono stati oggetto di pubblicazione:

Different mechanisms cause imprinting defects at the IGF2/H19 locus in Beckwith-Wiedemann syndrome and Wilms' tumour.
Cerrato F, Sparago A, Verde G, De Crescenzo A, Citro V, Cubellis MV, Rinaldi MM, Boccuto L, Neri G, Magnani C, D'Angelo P, Collini P, Perotti D, Sebastio G, Maher ER, Riccio A.
Hum Mol Genet. 2008 May 15;17(10):1427-35.

Inoltre nel corso del dottorato ho maturato una esperienza in campo bioinformatico, su un argomento non correlato a quello discusso in questa tesi, che è stato oggetto di pubblicazione:

In silico docking of urokinase plasminogen activator and integrins.
Degryse B, Fernandez-Recio J, Citro V, Blasi F, Cubellis MV.
BMC Bioinformatics. 2008 Mar 26;9 Suppl 2:S8.

SUMMARY

BWS is a developmental disorder characterized by variable clinical features, including overgrowth, macroglossia, abdominal wall defects and increased incidence of embryonal tumours that is caused by defective expression of imprinted genes located on chromosome 11p15.5.
A 1-Mb cluster of imprinted genes is present at chromosome 11p15.5.
The BWS cluster, which is well studied in the mouse, is functionally subdivided into two domains with an imprinting centre (IC) each. The genes including the paternally expressed Insulin-like Growth Factor 2 (*IGF2*) and the maternally expressed *H19* are controlled by IC1 that is located between the two genes. IC1 is postulated to be a DNA methylation sensitive chromatin insulator. On the maternal chromosome, its function is mediated by the binding of the insulator protein CTCF. On the paternal chromosome, methylation abolishes the CTCF binding allowing the activation of the *IGF2* gene by downstream enhancers.

The aim of my work has been to investigate the causes and mechanisms by which imprinting defect arises at the imprinting centre, in particular that involving IC1, in BWS.

We found gains of methylation defects at IC1 in 10% of the BWS cases.
In some of these patients, such epigenetic defect is associated with microdeletions in IC1. These mutations result in the hypermethylation of the remaining IC1 region, loss of IGF2/H19 imprinting and fully penetrant BWS phenotype when maternally transmitted.
However, some of these microdeletions result in the hypermethylation of the locus and some do not, suggesting that the type of target sites deleted is relevant for the phenotypic effect. To investigate the mechanisms by which these mutations influence the epigenetic status and function of the insulator, we have set up a cell culture system in which the different human IC1 alleles are transfected and analysed. In particular, the binding of CTCF to the mutant alleles is being analysed by ChIP while their barrier activity is analysed by using vectors in which these regions are placed between the SV40 enhancer and a luciferase (luc) expression cassette.
Our data indicate that the abnormally longer clusters of sites, characteristic of the alleles with microdeletion, reduce the affinity for CTCF and this results in gain of methylation of the locus and loss of insulator function.
Furthermore, we have investigated in more detail the DNA methylation of IC1 in patients without accompanying deletion to verify if methylation defect involves the entire IC1 region, and the timing at which imprinting defects occur

by determining the grandparental origin of the chromosome carrying the methylation defect in these BWS patients.
By analysing some patients with IC1 hypermethylation and no deletion, a detailed methylation analysis showed that the abnormal methylation at IC1 is mosaic in the majority of the cases and can affect either the entire or only the 3' half of the regulatory region.
The segregation of the chromosome with the imprinting defect in the healthy relatives excludes inherited mutations in the chromosome 11p15.5 region.
We observed that the imprinting defect derived from either the maternal grandfather or maternal grandmother chromosome. These results indicate that, in the absence of deletions, IC1 hypermethylation is unlikely to be the result of an incomplete erasure of the imprints, but rather suggest that the epigenetic defect arises later on, during imprint establishment in the gametes or imprint maintenance in early embryogenesis.
Considering the sporadic nature of these cases and the absence of association with the sequence in cis, it is possible that IC1 hypermethylation arises as consequence of stochastic events or as consequence of a genetic defect acting in trans.

Overall, these results indicate that methylation imprinting defects at the IGF2–H19 locus can result from inherited mutations of the IC1 that reduce the affinity for CTCF or from causes independent from the sequence in cis.
In the latter case, it is possible that the defect strikes a factor that acts in trans and that it is essential for the maintenance of the no methylated state. In both cases, the epigenetic abnormalities are usually present in the patients in the mosaic form and probably acquired by post-zygotic de novo methylation.

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